Weekly Patterns in Smoking Habits and Influence on Urinary Cotinine and Mutagenicity Levels: Confounding Effect of Nonsmoking Policies in the Workplace

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Abstract

Lifestyle factors such as smoking have been shown to influence urinary mutagenicity. Therefore, these factors have to be considered carefully when evaluating occupational genotoxic exposures. We investigated day-to-day variability in active and passive tobacco smoke exposure by studying urinary cotinine levels and determined their influence on observed urinary mutagenicity. Urinary cotinine was assessed for 105 subjects employed in the rubber manufacturing industry in the Netherlands on Sunday, Wednesday, and Thursday. Urinary mutagenicity was measured by the *Salmonella typhimurium* strain YG1041 with metabolic activation for the Sunday urine sample and a pooled weekday urine sample. A sharp decrease in urinary cotinine concentration was observed during the week compared to Sunday for smokers (39%; $P < 0.01$) and nonsmokers (23%). Different smoking habits on Sunday resulted in higher regression coefficients for categorical proxies for smoking habits and urinary mutagenicity levels. However, regression coefficients for urinary cotinine and urinary mutagenicity were similar for the Sunday and weekday urine samples ($\beta = 0.29$ and $\beta = 0.28$, respectively). Consequently, these estimates were used to adjust urinary mutagenicity for tobacco smoke intake. Cotinine-adjusted urinary mutagenicity levels were comparable between smokers and nonsmokers, and a similar increase in urinary mutagenicity of 39% and 34%, respectively, was observed for both smokers and nonsmokers due to occupational genotoxic exposures or other changes in lifestyle factors. These results indicate that the introduction of nonsmoking policies in the workplace has reduced exposure to mainstream and environmental tobacco smoke, resulting in a temporal variation in lifestyle-related mutagenicity. Therefore, adequate adjustment for daily tobacco smoke exposure is a necessity when using the urinary mutagenicity assay to evaluate possible genotoxic exposures in the workplace.

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Introduction

Urinary mutagenicity is often used as a biomarker of exposure to study the genotoxic effect of occupational exposures (1–4). Because of the nonselective character of the urinary mutagenicity assay, the assay has been found particularly useful in occupational settings with exposure to complex mixtures (5–9). However, the nonselective character of the assay makes it prone to confounding factors such as smoking and diet (10–13). Therefore, these factors have to be considered carefully, especially when genotoxic exposures are low (10). Because of the influence of lifestyle factors on urinary mutagenicity, large variations in background mutagenicity levels can be expected between subjects. Therefore, when comparing occupationally exposed and nonexposed groups, large samples are needed to ensure random distribution of confounding lifestyle factors. Another approach that has been used to control for variation in mutagenicity due to lifestyle factors is the use of the subjects as their own internal control. In this approach, the increment in urinary mutagenicity between urine samples collected before and after suspected mutagenic exposure is studied (14). The underlying assumption in this approach is that confounding lifestyle factors do not significantly change within subjects over time. However, differences exist in activity patterns during the weekend and weekdays such as more frequent visits to restaurants and bars and an increased number of active smokers in the personal environment. These factors have been shown to influence the exposure to MS[2] and ETS (15, 16).

The aim of the present investigation was to study the day-to-day variability in active and passive smoking by studying urinary cotinine levels and to determine their influence on observed urinary mutagenicity. Urinary mutagenicity was measured by the *Salmonella typhimurium* strain YG1041 with metabolic activation. S9 mix from aroclor induced rat livers was used as metabolic activation system to detect indirect mutagenic compounds or metabolites in urine.

Materials and Methods

Subjects

After completion of a survey, subjects ($n = 116$) were randomly selected based on their reported smoking habits and external genotoxic exposure profile from a group of 225 male

[2] The abbreviations used are: MS, mainstream smoke; ETS, environmental tobacco smoke.

Table 1 Mean urinary cotinine concentration (micrograms/gram of creatinine) and intra- and interindividual cotinine variability by smoking status and average number of cigarettes smoked per day

	Urinary cotinine								
	Sunday			Weekday samples (Wednesday and Thursday)					
	N[a]	GM[b]	GSD[c]	N[d]	GM	GSD	GSD_{intra}[e]	GSD_{inter}[f]	λ[g]
Nonsmokers	67	4.4	4.33	134	3.4	3.95	2.30	2.98	0.60
Smokers	38	1096.7	2.06	76	665.4	1.91	1.35	1.78	0.27
1–10 cigarettes/day	17	933.9	2.06	34	683.2	2.10	1.40	1.95	0.25
>10 cigarettes/day	21	1249.1	2.04	42	651.3	1.77	1.30	1.67	0.26

[a] Number of subjects.
[b] Geometric mean.
[c] Geometric SD.
[d] Number of samples.
[e] Estimated geometric SD of intraindividual distribution of the log-transformed cotinine concentrations.
[f] Estimated SD of interindividual distribution of the log-transformed cotinine concentrations.
[g] Variance ratio of the intra- and interindividual variance components.

subjects participating on a voluntary basis in a large exposure survey among nine companies in the rubber manufacturing industry in the Netherlands (17). Subjects were employed full time. All companies but one had a strict nonsmoking policy at the workplace, allowing smoking only during breaks in designated areas in the company.

Spot urine samples were collected on Sunday, Wednesday, and Thursday at approximately the same time of day (around 4 p.m.), stored in polyethylene containers, and kept at −20°C until use. Information regarding smoking status (yes/no) and average number of cigarettes smoked per day (0, 1–10, >10) was obtained by a self-administered questionnaire completed before the survey and by assessing urinary cotinine levels.

Analytical Procedures

Cotinine. Cotinine in urine was quantified by high-performance liquid chromatography according to the method of Barlow *et al.* (18), using the modifications described by Parviainen and Barlow (19). Urine samples with undetectable cotinine levels (limit of detection, 25 nmol/liter) were arbitrarily assigned a value of one-half of the detection limit. Creatinine levels were used to estimate urinary dilution by using a colometric test based on the Jaffé reaction between creatinine and sodium picrate. Cotinine levels were expressed in micrograms/gram of creatinine.

Mutagenic Activity. Urine samples collected on Wednesday and Thursday were pooled for each subject before mutagenicity analysis. A volume corresponding to 0.5 mmol of creatinine of the Wednesday and Thursday urine sample was pooled, resulting in a volume corresponding to 1 mmol of creatinine. Urine aliquots corresponding to 1.0 mmol of creatinine from the Sunday urine sample and a pooled weekday urine sample were neutralized to pH 7.0 and extracted with XAD-2 resin (6-cm^3 bed volume). After the urine was passed through the resin, the column was washed with distilled water, and the adsorbed material was eluted with 10 ml of acetone. After evaporation at 40°C under nitrogen, the residue was dissolved in 2.5 ml of DMSO (14, 20). Urine extracts were assayed for mutagenic activity with the *S. typhimurium* bacteria strain YG1041 with S9 mix of aroclor induced rat liver (20, 21). Mutagenic activity was calculated based on the dose-response curves acquired at different dose levels. The slope of the linear component was used as an estimate of the mutagenic potency (22). Urinary mutagenic activity levels were expressed in revertants/gram of creatinine.

Statistical Methods

Urinary cotinine and mutagenicity levels for both smokers and nonsmokers were log-normally distributed. Therefore, the natural logarithm of the cotinine concentration and mutagenic activity was used in all statistical procedures.

Intra- and interindividual variance components of urinary cotinine based on the Wednesday and Thursday urine samples were estimated using a one-way nested random effects ANOVA model. The influence of smoking habits and urinary cotinine levels on the mutagenic activity in urine was studied in separate linear regression models. Mean urinary cotinine concentration was calculated for the pooled weekday urine sample based on the cotinine levels of the Wednesday and Thursday urine samples. All statistical analyses were performed using SAS version 6.12 software (23).

Results

Of the 116 subjects, 105 subjects (90.5%) had complete data concerning smoking habits, urinary cotinine (Sunday, Wednesday, and Thursday urine samples), and mutagenic activity levels (Sunday urine sample and a pooled weekday urine sample). No systematic differences in smoking habits, urinary cotinine, and mutagenic activity levels were observed for subjects with incomplete data ($n = 11$) or for those with complete data ($n = 105$). Consequently, the results presented in this study are based on these 105 subjects. Subjects were all male, with a mean age of 37.9 ± 9.0 years.

A large statistically significant difference ($P < 0.0001$, t test) in mean urinary cotinine concentration between smokers and nonsmokers was observed for Sunday and weekday urine samples (Table 1; Fig. 1). Furthermore, a clear dose-response relationship was found between the average number of cigarettes smoked per day and urinary cotinine levels for smokers on Sunday. However, this dose-response relationship was not found for the weekday samples, in which almost no difference in mean urinary cotinine levels was observed for the different categories of average number of cigarettes smoked per day.

Analyses of the inter- and intraindividual variability in urinary cotinine levels of Wednesday and Thursday samples revealed an overall higher interindividual variability. Hence, the ratio (λ) of the intra- and interindividual variability for all smoking categories was well below 1, indicating that the observed cotinine levels were predominantly influenced by

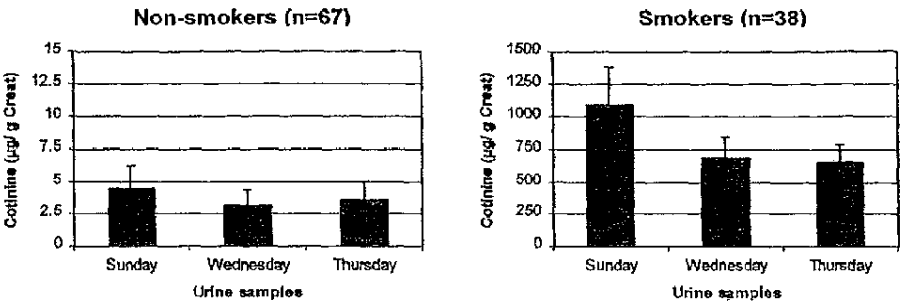

Fig. 1. Geometric mean and 95% upper confidence limit of urinary cotinine concentration (in micrograms/gram of creatinine) by smoking status and sampling day.

Table 2 Relationship between smoking status, average number of cigarettes smoked per day, and the natural logarithm of urinary cotinine levels and urinary mutagenicity levels on Sunday and weekdays

		Urinary mutagenicity			
		Sunday		Weekdays	
		β (SE)	R^{2a}	β (SE)	R^2
Model 1	Smoking[b]	1.63 (0.29)[c]	0.23	1.51 (0.23)[c]	0.29
Model 2	1–10 cigarettes/day[b]	1.24 (0.39)[c]	0.25	1.31 (0.31)[c]	0.30
	>10 cigarettes/day[b]	1.94 (0.36)[c]		1.67 (0.28)[c]	
Model 3	Urinary cotinine (μg/gram creatinine)	0.29 (0.05)[c]	0.26	0.28 (0.04)[c]	0.31

[a] Explained proportion of total variance.
[b] Tested against nonsmoking model; $\exp^{\beta}$ yields an estimate of the multiplicative effect of the corresponding factor on urinary mutagenicity.
[c] $P < 0.01$.

Table 3 Mean urinary mutagenicity unadjusted and adjusted for urinary cotinine concentrations by smoking status

	Sunday urinary mutagenicity (Rev/g creatinine)	Weekday urinary mutagenicity (Rev/g creatinine)	% increase in mutagenicity
	Mean ± GSD[a]	Mean ± GSD	%[b]
Unadjusted mutagenicity			
Nonsmokers	6752 ± 4.98	8641 ± 3.36	28%
Smokers	34436 ± 2.95	39148 ± 2.63	14%
Cotinine-adjusted mutagenicity[c]			
Nonsmokers	4416 ± 4.92	5913 ± 3.37	34%
Smokers	4664 ± 2.70	6470 ± 2.49	39%

[a] Geometric mean ± geometric SD.
[b] Percentage of difference between urinary mutagenicity levels on Sunday and weekdays.
[c] ln(cotinine-adjusted Sunday urinary mutagenicity) = ln(Sunday urinary mutagenicity) − 0.29 × ln(Sunday urinary cotinine concentration); ln(Cotinine adjusted Week urinary mutagenicity) = ln(Week urinary mutagenicity) − 0.28 × ln(weekday urinary cotinine concentration).

the individual. It is noteworthy that this phenomenon was more pronounced for smokers than for nonsmokers.

Smoking status (yes/no), categories of average number of cigarettes smoked per day, and urinary cotinine levels were clearly associated with urinary mutagenicity, with only minor differences in the explained proportion of the total variance for the different proxies used for smoking habits in these models (Table 2). However, an overall larger proportion of the total variability in urinary mutagenicity was explained by the proxies of smoking habits for weekday urine samples than for Sunday urine samples. Observed regression coefficients for smoking status and categories of average number of cigarettes smoked per day were higher for Sunday urine samples than for pooled weekday urine samples, with the exception of light smoking (1–10 cigarettes/day). Regression coefficients observed for the relationship between urinary cotinine levels and urinary mutagenicity for the Sunday and pooled weekday urine samples were practically identical ($\beta = 0.29$ and $\beta = 0.28$, respectively).

In Table 3, mean urinary mutagenicity levels of the Sunday and pooled weekday samples are presented. Smokers had, on average, a 5-fold higher urinary mutagenicity level than nonsmokers. Furthermore, both smokers and nonsmokers showed elevated levels of urinary mutagenicity when Sunday and pooled weekday urine samples were compared, with an increase of 28% for nonsmokers and 14% for smokers. However, cotinine-adjusted urinary mutagenicity levels (based on the observed relationship between urinary cotinine and urinary mutagenicity), revealed an almost identical increment in urinary mutagenicity for nonsmokers and smokers (34% and 39%, respectively). Hence, the observed levels of cotinine-adjusted urinary mutagenicity were almost similar for smokers and nonsmokers.

Discussion

Smoking is almost invariably the most important confounder or effect modifier in studies focusing on occupational and environmental genotoxic exposures. To control for tobacco smoke intake, several exposure indices have been used, including, for

example, the number of cigarettes smoked and several biochemical tests for plasma or saliva thiocyanate, expired carbon monoxide, and carboxyhemoglobin (24, 25). These exposure proxies have been found unsuitable because of a lack of sensitivity and specificity (26). Cotinine, one of the major metabolites of nicotine, has been considered as the most accurate biochemical indicator of exposure to MS and ETS (26, 27). Accordingly, urinary cotinine has been used in several studies to quantify the influence of tobacco smoke on urinary mutagenicity (9, 10, 28). However, other lifestyle factors such as diet have been shown to influence urinary mutagenicity as well (10). Because no biochemical indicators are available to control for all lifestyle factors, another approach has been advocated. In this approach, the studied subjects are used as their own internal control. The underlying assumption in this approach is that mutagenic exposure from other sources (lifestyle-related mutagenicity) does not vary temporarily (14). However, due to the introduction of strict nonsmoking policies at indoor workplaces in the Netherlands in the 1990s, this underlying assumption could possibly be refuted with regard to MS and ETS exposure. All companies but one in the present study had strict nonsmoking policies allowing smoking only at designated times and areas of the company.

We investigated the cotinine levels in urine collected on Sunday, Wednesday, and Thursday and observed a statistically significant decrease ($P < 0.01$, t test) for smokers in both unadjusted and creatinine-adjusted urinary cotinine levels during the week when compared to Sunday. Nonsmokers showed a similar downward trend, but the decrease did not reach statistical significance. Nevertheless, both downward trends in urinary cotinine levels suggest a decrease in both active and passive intake of tobacco smoke during the week compared to the weekend. Interestingly, heavy smokers (>10 cigarettes/day) showed a larger decrease in cotinine concentrations than light smokers (1–10 cigarettes/day), resulting in comparable cotinine concentrations during the week. Heavy smokers were probably more affected by nonsmoking policies in the workplace, resulting in a tobacco smoke intake comparable to that of light smokers during weekdays.

The low ratio of intraindividual:interindividual variability among active and passive smokers indicated a high degree of interindividual variability in cotinine concentration. Other investigators have found similar results, which presumably represent intersubject differences in nicotine metabolism and inhalation patterns (29, 30). However, because of the approximately 20-h half-life of cotinine, urinary cotinine levels of Wednesday and Thursday samples could potentially have been autocorrelated and thereby have resulted in an underestimation of the intraindividual variability (31).

Regression analysis between several qualitative estimates of MS exposure and urinary mutagenicity showed a clear relationship between active smoking and urinary mutagenicity. However, the observed association between urinary cotinine and urinary mutagenicity also implicates a relationship between passive smoking and urinary mutagenicity. Stratified analyses for smokers and nonsmokers revealed no significant difference in the observed regression coefficients (data not shown). Therefore, the overall observed relationship between urinary cotinine and urinary mutagenicity was used to adjust for MS and ETS exposure.

In the presented study, urinary mutagenicity on Sunday was used as an estimate of mutagenic exposure due to lifestyle factors, whereas urinary mutagenicity on weekdays was used as a measure of mutagenic exposure due to lifestyle factors and occupational exposure. The difference between these two measures would therefore yield an estimate of the mutagenic activity due to occupational genotoxic exposure. Adjustment of urinary mutagenicity levels for urinary cotinine concentrations revealed comparable background mutagenicity levels due to lifestyle factors other than tobacco smoke for smokers and nonsmokers, indicating adequate adjustment for tobacco smoke intake. Increases in mutagenicity due to workplace exposure or other changes in lifestyle factors, such as diet, were similar for smokers and nonsmokers after adjustment for MS and ETS exposure. Without adjustment for MS and ETS exposure, a different conclusion would have been reached. It is worth noting that adjusted urinary mutagenicity levels for smokers, although statistically nonsignificant, were still slightly elevated compared with those for nonsmokers. This could have been caused either by inadequate adjustment of urinary mutagenicity for cotinine levels or by smoking-induced enzyme systems leading to higher urinary mutagenicity levels due to mutagenic exposures from other sources (32–34).

These results indicate that the introduction of nonsmoking policies in the workplace has reduced exposure to tobacco smoke by active and passive smoking, resulting in a temporal variation in lifestyle-related mutagenicity. Therefore, adequate adjustment for daily tobacco smoke exposure is a necessity when using the urinary mutagenicity assay to evaluate possible genotoxic exposures in the workplace.

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